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### Review

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**7-ketocholesterol and 7 $\beta$ -hydroxycholesterol: in vitro and animal models used to characterize their activities and to identify molecules preventing their toxicity**

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**Running title:** *In vitro* and *in vivo* models to evaluate the activities of 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol

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#### Abbreviations used:

11 $\beta$ -HSD1: 11 $\beta$ -hydroxysteroid-dehydrogenase type I; 11 $\beta$ -HSD2: 11 $\beta$ -hydroxysteroid-dehydrogenase type II; 24S-OHC: 24S-hydroxycholesterol; 3,4,7-THSAP: 3,4,7-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione propionic acid; 3,4-DHSAP: 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione propionic acid; 3,4,7-THSA: 3,4,7-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 3,4-DHSA: 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 5 $\alpha$ , 6 $\alpha$ -epox: 5 $\alpha$ , 6 $\alpha$ - epoxy cholesterol; 5 $\beta$ , 6 $\beta$ -epox: 5 $\beta$ , 6 $\beta$ - epoxy cholesterol; 7-DHC: 7-dehydrocholesterol; 7-KC: 7-ketocholesterol; 7 $\alpha$ -OHC: 7 $\alpha$ -hydroxycholesterol; 7 $\beta$ -OHC: 7 $\beta$ -hydroxycholesterol; ACAT: Acyl-coenzyme A: cholesterol acyltransferases; ARMD: age-related macular degeneration; ARPE19: human retinal pigment epithelial cells; A $\beta$ : amyloid-beta; BAD: Bcl-2-associated death promoter; CAEC: carotid arterial endothelial cells; CASMC: coronary arterial smooth muscle cells; CH25H: cholesterol 25-hydroxylase; ChEH: cholesterol epoxide hydrolase; cPLA2 $\alpha$ : cytosolic phospholipase A2 alpha; DHA: docosahexaenoic acid; DMF: dimethylfumarate; EB12: Epstein-Barr virus-induced G-protein coupled receptor 2; EGCG: Epigallocatechin 3-gallate; ERK: extracellular signal-regulated kinases; fhRPE: human fetal retinal pigment cells; GPR: G-protein coupled receptor;

HAEC: human aortic endothelial cells; HA-VSMC: human aortic vascular smooth muscle aortic cells; HUVEC: human umbilical vascular endothelial cells; IP<sub>3</sub>: inositol 1,4,5-trisphosphate; iPSCs: inducible pluripotent stem cells; LCAT: lecithin-cholesterol acyltransferase; LDL: Low Density Lipoprotein; LPS: lipopolysaccharides; LXR: Liver X-receptor; MBP: Myelin Basic Protein; MS: multiple sclerosis; NRF2: nuclear factor erythroid 2-related factor 2; ORPL: oxysterol-binding protein related proteins; PBMC: peripheral blood mononuclear cells; PLP: Proteo Lipid Protein; RAEC: Rat aortic endothelial cells; ROS: reactive oxygen species; RPE: retinal pigment epithelium; RXR: Retinoïd X receptor; SC: *Saccharomyces cerevisiae*; SCFA: Short chain fatty acids; SERCA: sarco-/endo-plasmic reticulum Ca<sup>2+</sup>-ATPase; SM: smooth muscle; SOAT1: sterol O-acyltransferase 1; SREBP: sterol regulatory element binding protein; SULT: sulfation via sulfotransferase; TP: *Tetrahymena pyriformis*; VCAM-1: vascular cell adhesion molecule 1; VGCC: L-type voltage-gated Ca<sup>2+</sup>-channels; X-ALD: X-linked adrenoleukodystrophy;

## Abstract

Oxysterols are molecules derived by the oxidation of cholesterol and can be formed either by auto-oxidation, enzymatically or by both processes. Among the oxysterols formed by auto-oxidation, 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol are the main forms generated. These oxysterols, formed endogenously and brought in large quantities by certain foods, have major cytotoxic properties. They are powerful inducers of oxidative stress, inducing dysfunction of organelles (mitochondria, lysosomes and peroxisomes) that can cause cell death. These molecules are often identified in increased amounts in common pathological states such as cardiovascular diseases, certain eye conditions, neurodegenerative disorders and inflammatory bowel diseases. To oppose the cytotoxic effects of these molecules, it is important to know their biological activities and the signaling pathways they affect. Numerous cell models of the vascular wall, eye, brain, and digestive tract have been used. Currently, to counter the cytotoxic effects of 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol, natural molecules and oils, often associated with the Mediterranean diet, as well as synthetic molecules, have proved effective *in vitro*. Bioremediation approaches and the use of functionalized nanoparticles are also promising. At the moment, invertebrate and vertebrate models are mainly used to evaluate the metabolism and the toxicity of 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol. The most frequently used models are mice, rats and rabbits. In order to cope with the difficulty of transferring the results obtained in animals to humans, the development of *in vitro* alternative methods such as organ / body-on-a-chip based on microfluidic technology are hopeful integrative approaches.

**Keywords:** 7-ketocholesterol, 7 $\beta$ -hydroxycholesterol, cell models, animal models, microfluidic, signaling pathways.

## 1 – Introduction

At the moment, there are several lines of evidence in numerous cell lines from different species that 7-ketocholesterol (7KC) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OHC) induce cytotoxic side effects including rupture of RedOx homeostasis, inflammation and cell death defined as oxiaapoptophagy (OXIdative stress + APOPTOsis + autoPHAGY). This is associated with important modifications of the lipid profile and with organelle dysfunction (mitochondria, lysosome, peroxisome) [1]. As increased levels of 7KC and 7 $\beta$ -OHC are associated in major diseases such as age-related diseases (cardiovascular diseases, neurodegeneration, eye disorders, osteoporosis and some cancers) and chronic diseases (bowel diseases) [2, 3], there is a need to better understand the biological activities of these molecules not only *in vitro* but also *in vivo* in order *i)* to identify pharmacological targets, and *ii)* to identify natural and synthetic compounds capable of counteracting 7KC- and 7 $\beta$ -OHC-induced side effects, as well as *iii)* to develop innovative therapeutic strategies (targeted nanotherapies [4], bioremediation [5, 6]). To overcome deficits in animal models whose results are sometimes difficult to transfer to humans, the development of alternative methods (“Lab on a Chip” / “Body on Chip” / organoids), perhaps combined with the use of inducible pluripotent stem cells (iPSCs), are promising integrative approaches.

The present review will discuss the biogenesis and catabolism of 7KC and 7 $\beta$ -OHC; the biological activities of these oxysterols; their signaling pathways associated with oxidative stress, organelle dysfunction, cell death and inflammation; and the natural and synthetic compounds capable of preventing or attenuating their cytotoxic effects. The *in vitro* and *in vivo* models used to demonstrate these effects will be presented. Potentially suitable alternative methods to animal models (“Lab on a Chip” / “Body on Chip” / organoids / iPSCs) allowing to study the biological activities of 7KC and 7 $\beta$ -OHC and to identify molecules capable to modulate their biological activities will also be discussed.

## 2 - Biogenesis and catabolism of 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol

Sterols are isoprenoid-derived molecules that have essential functions in eukaryotes, and in higher plants. Oxysterols are oxidized derivatives of cholesterol or its precursors post lanosterol [7-9]. They are associated with numerous major pathologies: cardiovascular diseases, neurodegeneration, eye disorders, osteoporosis and some cancers [2, 3, 10]. The most well-

known oxysterols are oxygenated derivatives of cholesterol which are formed by the addition of one or more oxygenated functional groups to 27-carbon cholesterol molecule [11]. Higher plants, algae, most fungi, and vertebrates synthesize sterols whereas insects do not. In vertebrates, cholesterol is the major sterol, whereas a mixture of various sterols, named phytosterols, is present in higher plants with sitosterol [(24R)-24-ethyl cholesterol] usually predominating. The phytosterols, which are structurally similar to cholesterol, can also be oxidized to give oxyphytosterols such as 7-ketositosterol and 7 $\beta$ -hydroxysitosterol [12]. Normocholesterolemic healthy individuals contain a mixture of oxysterols in their peripheral blood, accounting for 1–5% of total cholesterol [13]. Cholesterol oxidation can occur on the hydrocarbon rings (A, B, C and D) of the steroid nucleus or on the side chain of the cholesterol molecule [11]. The oxysterols can be obtained by auto-oxidation, enzymatically or by both processes [11, 14-17]. Oxysterols formed enzymatically are often oxidized on the lateral chain and produced by cytochrome P450 enzymes with the exception of 25-hydroxycholesterol, which is formed by cholesterol 25-hydroxylase (CH25H) [11, 13]. For the latter, there are also arguments in favor of its formation by auto-oxidation of cholesterol [18, 19]. The double bond present on the B ring is the most energetically favorable target of free radical attack and therefore carbon atoms at the positions of 4, 5, 6 and 7 are more susceptible to free radical oxidation [15, 17]. Commonly, B-ring oxidized oxysterols include hydroxylated compounds: 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OHC) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OHC); oxysterols with a ketone group [7-ketocholesterol (7-KC, also named 7-oxocholesterol)]; epoxy cholesterol [5 $\beta$ , 6 $\beta$ - epoxy cholesterol (5 $\beta$ , 6 $\beta$ -epox), 5 $\alpha$ , 6 $\alpha$ -epoxy cholesterol (5 $\alpha$ , 6 $\alpha$ -epox)] and cholestan-3 $\beta$ , 5 $\alpha$ , 6 $\beta$  triol. Among the oxysterols oxidized at C7 [7 $\alpha$ -OHC (Systemic name: Cholest-5-en-3 $\beta$ ,7 $\alpha$ -dio; CAS (Chemical Abstracts Service) number: 566-26-7; lipid map: LMST01010013); 7 $\beta$ -OHC (Systemic name: 5-Cholestene-3 $\beta$ ,7 $\beta$ -diol; CAS number: 566-27-8; lipid map: LMST01010047) and 7KC (Systemic name: 7-oxo-Cholest-5-en-3 $\beta$ -ol; CAS number: 566-28-9; lipid map: LMST01010049)] (**Figure 1**). 7 $\alpha$ -OHC is formed enzymatically by the enzyme cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), and is a precursor of bile acids [20, 21].

The oxysterols 7KC and 7 $\beta$ -OHC can be ingested in certain industrial foods [22-24] and are present in high amounts in atheromatous plaques, oxidized lipoproteins and in the retinal drusen in patients with cardiovascular diseases and age-related macular degeneration (ARMD),



respectively. It has long been considered that these oxysterols were only formed by auto-oxidation of cholesterol [25]. There are also arguments supporting that exogenous 7KC is rapidly metabolized and excreted by the liver, suggesting that dietary 7KC make little or no contribution to atherogenesis [26]. At the moment, several putative mechanisms of cholesterol auto-oxidation are proposed [15, 16, 27, 28]. In cardiovascular diseases and ARMD, 7KC and 7 $\beta$ -OHC can be formed *in situ* under a pro-oxidant environment [14, 29-31]. The proportion of oxysterols in plasma of hypercholesterolemic patients is about 7KC (57%) > 7 $\beta$  / $\alpha$ -OHC (21%) > 5 $\beta$ ,6 $\beta$ -cholesterol epoxide (12%) > Triol (10%) while in atherosclerotic plaques it is about 7-KC (55%) > Triol (13%) > 7 $\alpha$  / $\beta$  -OHC (12%) > 5 $\beta$ ,6 $\beta$  / 5 $\alpha$ ,6 $\alpha$  -cholesterol epoxide (10%) [32]. Adverse environmental conditions, such as chronic air pollution exposure, have also been reported to favor 7-KC accumulation, foam cell formation, and atherosclerosis in mice [33].

Currently, it is also well established that 7KC can be formed via the CYP7A1 enzyme from 7-dehydrocholesterol (7-DHC), which is the precursor of Vitamin D3 (cholecalciferol), and which is present in high amounts in the plasma of patients with Smith Lemli Opitz syndrome who are deficient in the enzyme 7-dehydrocholesterol reductase (DHCR7) [21, 34]. It has also been demonstrated that the 11 $\beta$ -hydroxysteroid-dehydrogenase type I (11 $\beta$ -HSD1 / gene HSD11B1), which is responsible for the conversion of cortisone in cortisol, is also responsible for the conversion of 7-KC in 7 $\beta$ -OHC [21, 35-37]. In humans, 11 $\beta$ -HSD1 can also catalyse the conversion of 7 $\beta$ -OHC into 7KC [38]. In addition, the 11 $\beta$ -hydroxysteroid-dehydrogenase type II (11 $\beta$ -HSD2 / gene HSD11B2), which is responsible for the conversion of cortisol in cortisone, is also responsible for the conversion of 7 $\beta$ -OHC in 7KC [21, 35, 39]. Of note, the activity and reaction direction of 11 $\beta$ -HSD1 can be altered under conditions of 7-oxysterol excess, and could impact upon the pathophysiology of obesity [40, 41]. How 7KC and 7 $\beta$ -OHC are further metabolized is of great interest since the resulting metabolites may have important biological activities. Oxysterols are present *in vivo* in different forms, namely, the esterified, sulfated, and conjugated forms, as well as free oxysterols [42]. There are now several arguments supporting that 7KC can give rise to numerous metabolites, which probably vary depending on the cell type considered [21, 43]. However, little is known about the catabolism of 7 $\beta$ -OHC. However, for this oxysterol as well as for 7KC, regulation of its activity by sulfation via sulfotransferase 2B1b (SULT 2B1b) cannot be excluded [44]. In addition, potential modifications of the activities of

7KC and 7 $\beta$ -OHC by esterification can also occur. Thus, in human retinal pigment epithelial cells (ARPE19), it has been reported that the esterification of 7KC to fatty acids involves the combined action of cytosolic phospholipase A2 alpha (cPLA2 $\alpha$ ) and sterol O-acyltransferase (SOAT1) [45]. In addition, Acyl-coenzyme A, a cholesterol acyltransferase (ACAT, also abbreviated as SOATs) which converts cholesterol to cholesteryl esters and which play key roles in the regulation of cellular cholesterol homeostasis, is known to metabolize diverse substrates including both sterols and certain steroids [46]. Oxysterols are substrates of SOAT1 and SOAT2. Of note, in human promonocytic U937 cells, none of the cytotoxic effects observed with 7 $\beta$ -OHC and 7KC were noted with cholesterol, 7 $\beta$ -OHC-3-oleate and 7KC-3-oleate, with the exception of a slight increase in superoxide anion production with 7 $\beta$ -OHC-3-oleate [47]. The enzyme lecithin-cholesterol acyltransferase (LCAT) also permits synthesis of monoesters of 24(S)-hydroxycholesterol [48]. LCAT could also esterify all oxysterols as monoesters (3 $\beta$ -hydroxyl group) and it has been suggested that it could also generate diesters (3 $\beta$ -hydroxyl group and 27-hydroxyl group) in some cases [49]. Specifying the impact of these previously cited enzymes on 7KC and 7 $\beta$ -OHC, in diseases where the levels of these oxysterols are modified, could lead to the identification of new therapeutic targets. It has also been reported on human hepatoma cells (HepG2) that the mitochondrial sterol 27-hydroxylase (CYP27A1) was responsible from the 27-hydroxylated product of 7KC [50].

## **2 – *In vitro* study of the biological activities of 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol and identification of molecules modulating their activity**

The toxicity of certain oxysterols, such as 7KC and 7 $\beta$ -OHC, and the suspected mutagenicity of some of them, such as 5,6-epoxides [51, 52], led to study the toxic and mutagenic properties of these molecules in different cell models. In acidic aqueous solution, 5,6-epoxides can give cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (CT) as a single product [53]. Using the Ames test, no mutagenic activity of 7KC, 7 $\beta$ -OHC and 5,6-epoxides was found whereas CT was slightly mutagenic [53, 54]. However, 5,6-epoxides were mutagenic at high concentration on V79 Chinese hamster lung fibroblasts and induced the transformation of murine embryo cells [55, 56]. In addition, no genotoxicity was observed when CHO and Indian Muntjac fibroblastic cells were exposed to 5,6-epoxides [57]. Based on these different studies, it is currently considered unlikely that 5,6-epoxides will be direct carcinogenic [58]. As atherosclerosis is the first pathology in which the

contribution of oxysterols, mainly 7KC and 7 $\beta$ -OHC, was suspected several works were realized in this field [25, 42, 59]. Subsequently, due to several studies suggesting a role of 7KC and / or 7 $\beta$ -OHC in certain ocular diseases (cataract, ARMD), neurodegeneration (Alzheimer's disease in particular) [3], and bowel diseases [2], several types of cells from the eye, brain and digestive tract were used to characterize the cytotoxicity of 7KC and 7 $\beta$ -OHC and to identify molecules capable of preventing their side effects.

### ***2.1 - In vitro models applied to the study of cardiovascular diseases and the identification of cytoprotective compounds in this field***

Atherosclerosis, which favors thrombosis, stroke and myocardial infarction, is a slow multifactorial degenerative process involving inflammatory, oxidative and cytotoxic processes. In the development of atherosclerosis, which is a frequent age-related disease [3], major roles are attributed to 7KC and 7 $\beta$ -OHC identified at high levels in the plasma and atheroma plaques of atheromatous patients [60]. In order to determine the biological activities of these molecules, several cellular models have been used especially endothelial cells, smooth muscle cells and monocytic cells.

With regard to cellular models contributing to vessel structure, endothelial cells were used like the human umbilical vascular endothelial cells (HUVEC) [61] and some endothelial cell lines: a) human endothelial cells Eahy926 [62], b) human aortic endothelial cells (HAECs) [63, 64]; c) human endothelial cell line ISO-HAS [65], d) mouse endothelial cells (EOMA cells) [66], e) mouse carotid arterial endothelial cells (CAECs) [67]. The *in vitro* model of arterial relaxation of aortic rings was also used to study the impact of 7-hydroxycholesterols (7KC, 7 $\beta$ -OHC) on endothelial functions [68]. Endothelial cells derived from a primary rat aorta culture (rat aortic endothelial cells (RAEC)) were also used [69].

Rat A7r5 smooth muscle cells and human aortic vascular smooth muscle aortic cells (HA-VSMC) were used to assess the cytotoxic effects of 7-KC and/or 7- $\beta$ OHC [70, 71]. Smooth muscle cells can also be extracted from aortic tissue of patients [72]. Data obtained on primary cultures from mice (mouse coronary arterial smooth muscle cells (CASMCs) [73] or rabbit [74]) have also been reported.

For inflammatory cells, the most commonly *in vitro* models used are monocytic / macrophage cells THP-1, U937, J774, but also RAW 264.7 and preloaded macrophages (P388D1 cell line) [47, 75-81]. Monocytes were also cultured from human peripheral blood mononuclear cells (PBMCs) [82, 83] or harvested from the mouse peritoneal fluid (peritoneal macrophages) or from mouse aorta (arterial macrophages) [84].

Cardiomyocytes were studied using mouse cardiac cells isolated from HL1-NB mice [85].

These different cell models were not only used to study the impact of 7KC and 7 $\beta$ -OHC on the cells of the vascular wall, but also to identify natural and synthetic molecules capable of preventing the cytotoxicity of 7-KC and 7- $\beta$ OHC. In these cell models, the following data have been reported: Vitamin E ( $\alpha$ -tocopherol) inhibits 7KC-induced oxidative stress, and apoptosis [86, 87]; butyrate, which belongs to a family of gut microbial metabolites, as well as short chain fatty acids (SCFAs) decrease the formation and the activation of Nlrp3 inflammasome induced by 7KC [66]; epigallocatechin 3-gallate (EGCG) inhibits 7KC-induced monocyte–endothelial cell adhesion [65]; indicaxanthin, a bioactive pigment from cactus pear fruit, inhibits 7KC-induced THP-1 cell apoptosis [75]; soy-leaf extract has atheroprotective effects via modulation of Krüppel-Like Factor 2 and adhesion molecules in 7KC-treated HUVECs [88]; retinoid X receptor  $\alpha$  (RXR $\alpha$ ) modulator, K-80003, a non-steroidal anti-inflammatory drug, inhibits 7-KC-induced RXR $\alpha$  cytoplasmic translocation [79]; terculic acid, cyclopropene fatty acid, antagonizes 7KC-mediated inflammation [89]; azelnidipine, a calcium channel blocker, inhibits ROS-dependent expression of vascular cell adhesion molecule 1 (VCAM-1) induced by 7KC [90] Aronox, an anthocyanin-rich extract from *Aronia melanocarpa* E, inhibits apoptosis, ROS generation and the fall of the transmembrane mitochondrial potential induced by 7 $\beta$ -OHC [91]; dimethyl sulfoxide also prevents lysosomal and mitochondrial membrane permeabilization and ROS overproduction induced by 7 $\beta$ -OHC [92].

## ***2.2 - In vitro models applied to the study of eye diseases and the identification of cytoprotective compounds in this field***

The eye is composed of different regions, from the cornea to the retina, allowing it to perform its visual function. The retina is the combination of the neurosensory retina and the retinal pigment

epithelium [93]. The neural retina is composed of several cell types, including the glia, whose role is crucial for cholesterol biosynthesis. Cholesterol is the main sterol in the retina. It is present at the cellular level mainly as free cholesterol, but as cholesterol esters in the extracellular environment of the Bruch membrane. The accumulation of cholesterol esters at this level is a marker of retinal aging and decreased retinal function. Subject to dietary influence and local synthesis, cholesterol is also regulated through its interactions with the different cell populations of the retina, and in particular those that involve neuron-glia communication. Age-related macular degeneration (ARMD) is associated with the development of abnormal macromolecular deposits called drusen. These are located in the Bruch membrane [93]. Recent investigations concerning the nature of these deposits, their relationship to inflammatory and immune reactions and the important role of oxidative stress have broadened the hypotheses on the pathophysiological mechanisms leading to ARMD lesions. Many analogies with the mechanisms involved in the genesis of atherosclerosis lesions suggest physiopathological similarities between these two diseases [93]. Currently, several data support the concept that oxysterols, mainly 7KC, are involved in the genesis of ARMD leading to cytotoxic, pro-oxidant and pro-inflammatory activities [14, 94]. In addition, some oxysterols have been identified by gas chromatography in human cataracts obtained after surgery: 7KC, 7 $\beta$ -OHC, 5 $\alpha$ , 6 $\alpha$ -epoxycholestanol, 20 $\alpha$ -hydroxycholesterol, and 25-hydroxycholesterol [95]. In ARMD, 7KC seems to activate several kinase signaling pathways via multiple transcription factors to induce cytokines and intracellular effectors causing cell death [96].

Among the ocular pathologies studied, several of them concern ARMD and cataracts or vision disorders associated with different pathologies such as Parkinson's disease or Smith Lemli Opitz syndrome [16, 25, 93]. Several *in vitro* models have been used to study the effects of oxysterols on the eye. The most common models are retinal pigment epithelial cells: human ARPE-19 cells [97-100], fetal human retinal pigment epithelial cells (prepared from eyes of human fetuses) [98] or those isolated from ox eyes [101]. Primary cultures of porcine retinal pigment epithelial cells isolated from pigs eyes are also used [102]. Rat retinal precursor cell line R28 has been used less frequently [103]. Microglial retinal cells have also been isolated to study the neuroinflammation phenomena that occur in the outer retina [98, 104]. In addition, 661W photoreceptor cell lines were used [104, 105].

Currently, two molecules were reported to inhibit the toxic effects of 7KC and 7 $\beta$ -OHC on retinal epithelial cells. Sterculic acid, which is a cyclopropene fatty acid, antagonizes 7KC-mediated inflammation and inhibits choroidal neovascularization [89]; and resveratrol, a polyphenol belonging to the stilbene class used in the treatment of ARMD attenuate 7KC-induced cell death and prevent 7KC-induced VEGF secretion [106].

### ***2.3 - In vitro models applied to the study of neurodegenerative diseases and the identification of cytoprotective compounds in this field***

Since increased levels of 7KC and 7 $\beta$ -OHC were identified in the plasma and/or the cerebrospinal fluid of patients with demyelinating and non-demyelinating neurodegenerative diseases such as multiple sclerosis (MS) [107], X-linked adrenoleukodystrophy (X-ALD) [108], and Niemann-Pick disease [21], the effects of 7KC and 7 $\beta$ -OHC were evaluated on different types of nerve cells (neurons, glial and microglial cells). In these cells, several studies addressed the question of whether these oxysterols can induce organelle dysfunction (mitochondria, lysosome, peroxisome), oxidative stress, metabolic dysfunction, and cell death, which are hallmarks of neurodegeneration.

Thus, undifferentiated and retinoic acid-differentiated human SH-SY5Y neuroblastoma cells were used to study the biological ability of certain oxysterols such as 24S-hydroxycholesterol (24S-OHC). In these cells, 7KC-induced cell death was attenuated by 24S-OHC [109]. Human SK-N-BE neuroblastoma cells were also treated with 7KC at the physiopathological concentration of 1  $\mu$ M with the aim of comparing the effects of 7KC and 24S-OHC on tau phosphorylation, and to study the abilities to modulate the SIRT1-dependent neuroprotective pathway [110]. SK-N-BE cells were also used to compare the cytotoxicity of 7KC and 7 $\beta$ -OHC. In SK-N-BE cells, 7KC and 7 $\beta$ -OHC induce a loss of mitochondrial activity, an inhibition of cell growth, ROS overproduction, an enhancement of antioxidant enzymes activities, and a slight increase in Ca<sup>2+</sup> [111]. To determine how 7 $\beta$ -OHC could contribute to the development of Alzheimer's disease, the interaction between 7 $\beta$ -OHC and amyloid-beta (A $\beta$ ), whose extracellular accumulation in neuritic plaques is one of the hallmarks of Alzheimer's disease, was studied [112]. It was reported that 7 $\beta$ -OHC enhances the binding of A $\beta$  to these cells by up-regulating the expression and the synthesis of CD36 and  $\beta$ 1-integrin receptors [112]. In rat

pheochromocytoma PC12 cells exposed to sub-lethal concentrations of  $7\beta$ -OHC, a significant increase of cellular glutathione levels and an enhancement of cell tolerance against the subsequent oxidative stress were observed [113]. C6 rat glioma cells were also incubated with  $7\beta$ -OHC for 24 h with the purpose of evaluating its cytotoxicity and to determine the type of cell death induced by this oxysterol [114]. In these cells, it was demonstrated that  $7\beta$ -OHC induces a non-apoptotic mode of cell death associated with survival autophagy [114].

Immortalized murine glial cells (oligodendrocytes 158N) and microglial cells (BV-2) were also used to study the cytotoxic effects of 7KC and  $7\beta$ -OHC in the context of demyelinating and non-demyelinating neurodegenerative diseases. Indeed, these cells are suitable models to analyse oxidative stress, inflammation and cell death (apoptosis and autophagy) and to study the relationships between these different side effects [1, 115-117]. These cells constitute good models to study the impact of 7KC and  $7\beta$ -OHC on organelle structure, topography, functions and interactions (mitochondria, lysosome and peroxisome) [1]. They also permit to study the part taken by the peroxisome in cell death, as well as to identify natural or synthetic molecules capable of counteracting or attenuating 7KC- and  $7\beta$ -OHC-induced cytotoxicity.

To precisely define the impact of neuroinflammation in MS, organotypic hippocampal slice cultures were incubated with BV-2 cells without and with lipopolysaccharides (LPS) [118]. Under these conditions, an accumulation of 7KC was observed in brain tissue, and an induction of apoptosis associated with oxidative stress was reported in the BV-2 cells [118]. This phenomenon involves the translocation of NF- $\kappa$ B and the activation of poly(ADP-ribose)polymerase-1 (PARP-1), which is essential for microglial migration and consecutively regulates the expression of the iNOS, CD11a, and ICAM-1. These latter mechanisms are essential for the damaging activity of microglial cells [118]. Use of murine microglial BV-2 cells permitted demonstration of the ability of oleic acid (C18:1 n-9) and docosahexaenoic acid (DHA; C22:6 n-3), two major fatty acids present in the Mediterranean diet, to attenuate 7KC-induced oxiaoptophagy, associated with oxidative stress and with apoptotic and autophagic characteristics [1, 117, 119]. Currently, 158N cells are a widely used model to study the cytotoxicity of 7KC and  $7\beta$ -OHC, and to determine the effects of these oxysterols on major myelin proteins expressed by these cells (Myelin Basic Protein (MBP), Proteo Lipid Protein (PLP)) [115]. In 158N cells, anti-apoptotic and anti-oxidant capacities of natural or synthetic

compounds and their ability to attenuate or to prevent toxicity of 7KC or 7 $\beta$ -OHC were often evaluated. In 158N cells incubated with 7KC or 7 $\beta$ -OHC, the cytoprotective effect of dimethylfumarate (DMF; Tecfidera) and biotin (vitamin B8), which are used for the treatment of MS, have been shown [120, 121]. Natural products including argan oil, milk thistle seed oil, sea urchin egg oil, *Carpobrotus edulis* ethanol-water extract also strongly attenuate 7KC and/or 7 $\beta$ -OHC-induced cytotoxicity [121-125].

### **2.3 - *In vitro* models applied to the study of bowel diseases and the identification of cytoprotective compounds in this field**

Oxidative stress is thought to play a key role in the development of intestinal damage in inflammatory bowel disease, because of its primary involvement in intestinal cells aberrant immune and inflammatory responses to dietary antigens and to the commensal bacteria [126, 127].

In this context, human intestinal Caco-2 cells are often used and cultured in the presence of an oxysterols mixture (7KC, 5 $\alpha$ ,6 $\alpha$  epoxycholesterol, 5 $\beta$ ,6 $\beta$ -epoxycholesterol, 7 $\alpha$ -hydroxycholesterol and 7 $\beta$ -OHC) [127]. In this cell model pro-oxidant and pro-inflammatory effects of the oxysterol mixture were shown [128]. These cytotoxic effects were prevented by wine polyphenols [129, 130], olive oil polyphenols [131] and cocoa bean shells [128].

### **2.4 - Interest of the yeast model, *Saccharomyces cerevisiae* and of the protozoa model, *Tetrahymena pyriformis*, to study 7-ketocholesterol- and 7 $\beta$ -hydroxycholesterol-induced cytotoxicity**

The yeast, *Saccharomyces cerevisiae* (SC), is an excellent model for studying autophagy [132]. Due to the ability of 7-KC and 7 $\beta$ -OHC to induce oxiaoptophagy [119, 133], the SC model should provide a better understanding of the characteristics of autophagy induced by 7KC and 7 $\beta$ -OHC. In addition, the SC model is a well recognized for studying the peroxisome, which still remains a poorly characterized organelle [134]. The SC model should make it possible to specify the impact of 7-KC and 7 $\beta$ -OHC on the peroxisome in terms of biogenesis and function, peroxisome-mitochondria interaction [135]. The protozoan model *Tetrahymena pyriformis* (TP) is an interesting tool for performing environmental toxicology analyzes [136]. Compared to



commonly used cellular models, it is a reference model to address the impact of molecules on the phagocytosis of nano- or microparticles; on mobility (this protozoan moves thanks to numerous cilia); and on the relationships between organelles (mitochondria, peroxisome) and mobility. The TP model has been successfully used for the identification of cactus (*Opuntia ficus indica*) extracts capable of inhibiting 7KC-induced cell death [137]. These easy-to-implement cellular models are also interesting tools for identifying molecules that modulate the activities of 7-KC and 7 $\beta$ -OHC.

### ***2.5 – 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol signaling pathways based on in vitro studies***

The pathways involved in 7KC and 7 $\beta$ -OHC signaling for oxidative stress, and cell death, characterized by apoptotic and autophagic criteria (oxiaptophagy), and in 7KC- and 7 $\beta$ -OHC-induced inflammation (cytokine secretion, enhancement of adhesion molecule expression) have been primarily determined on the cellular models discussed in the preceding section. It is important to emphasize that these signaling pathways are highly conserved from one cell type to another and seem to be independent of the species considered [25, 87].

#### ***2.5.1 - 7-ketocholesterol-induced oxiaptophagy and inflammation: associated signaling pathways***

The schematic signaling pathways presented in **Figure 2** summarize the data obtained with 7KC in cells from different types and species: promonocytic / monocytic human U937 / THP1 cells [25, 47]; wild type human mammary tumor MCF-7 cells (caspase-3 deficient), and genetically modified MCF-7 (MCF-7/c3: stably transfected with caspase-3) [138], human retinal pigment epithelial cells (ARPE-19) [97, 139] and rat R28 retinal neurosensory cells [140]; murine oligodendrocytes 158N and murine microglial BV-2 cells [3, 119, 141-145].

In A7r5 rat aortic smooth muscle cells and murine oligodendrocyte 158N cells, 7KC has been shown to accumulate in lipid rafts [141, 146]. In U937 and BV2- cells, and based on artificial membrane models, 7KC interacts with plasma membrane phospholipids leading to important changes in membrane properties [25, 117, 147]. In U937, 158N and BV-2 cells, 7KC induces a mode of cell death defined as oxiaptophagy [47, 133]. 7KC stimulates an overproduction of ROS: superoxide anion ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ) [25]. In human aortic smooth muscle cells, 7KC activates the NADPH oxidase (NOX-4) via endoplasmic reticulum stress

involving the IRE-1/JNK/AP-1 signaling pathway which contributes to overproduction of ROS, leading to decreases in  $\Delta\Psi_m$  and apoptosis [148]. 7KC-induced apoptosis is characterized by an early externalization of phosphatidylserine [149] and by the following events: (i)  $Ca^{2+}$  influx; (ii) activation of calmodulin and calcineurin leading to BAD dephosphorylation and subsequent mitochondrial depolarization; (iii) mitochondrial release of cytochrome c, AIF and Endo-G; (iv) activation of caspase-2, -3, -7, -8, and -9; (v) truncation of BID; (vi) lower BCL-2 levels; (vii) cleavage of PARP; and (viii) of the DNA fragmentation factor (DFF45)/ICAD leading to the activation of caspase activated DNase (CAD) involved in internucleosomal DNA fragmentation [71, 138, 150-152]. In addition, 7KC-induced cell death is associated with an activation of the P2X7 receptor (involved in  $Na^+/Ca^{2+}$  influx and  $K^+$  efflux) [97, 143]. In 158N and BV-2 cells, 7KC also triggers an increased level of the voltage-gated  $K^+$  ( $K_v$ ) channel kv3.1b (involved in  $K^+$  efflux) protein, and an intracellular accumulation of  $K^+$  [143, 144]. The cytoplasmic accumulation of  $K^+$  is positively correlated with increased plasma membrane permeability to PI, ROS overproduction and loss of  $\Delta\Psi_m$  [143]. In monocytes macrophages (U937, RAW264.7, P388D1) and smooth muscle cells, it has been shown that 7KC inhibits the PDK-1/(Akt/PKB) signaling pathway [86, 153] and triggers the formation of multilamellar cytoplasmic structures named myelin figures [25, 154]. The ability of 7KC to induce also lysosomal modifications [122, 133, 155], formation of monodansyl cadaverine positive structures [156] and activation of LC3-I into LC3-II, also support that 7KC is capable of triggering autophagy. It is suggested that 7KC could induce a protective form of autophagy [73, 157] and it is now considered that myelin figures could be ultrastructural features of reticulophagy. Under treatment with 7KC, peroxisomal dysfunction (decreased peroxisomal mass suggested by decreased expression of ABCD3, altered peroxisomal  $\beta$ -oxidation supported by an accumulation of very long chain fatty acids) has also been reported [158-160]]. Whereas the part taken by the peroxisome in cell death is not well known [161], it has been demonstrated that peroxisomal dysfunctions are able to trigger oxidative stress [162, 163]. As the peroxisome is tightly connected with the mitochondria [164, 165] which plays key roles in 7KC-induced apoptosis [166], the contribution of the peroxisome to 7KC-induced cell death, which is associated with a rupture of the RedOx homeostasia, cannot be excluded [1].

It is also now well established that 7KC is a potent inducer of inflammation. This oxysterol increases cytokine secretion (IL-8, IL-1 $\beta$ ) and overexpression of adhesion molecules (VCAM-1,

ICAM-1, E-selectin). However, in U937 and THP-1 cells, the secretion of IL-1 $\beta$  and IL-8, and the expression of VCAM-1, ICAM-1, and E-selectin was lower than with 7 $\beta$ -OHC [61, 167, 168]. Currently, limited data are available on the metabolic pathway(s) contributing to 7KC-induced inflammation. In cultured ARPE-19 cells, 7KC-induced inflammation is mediated mostly through the TLR4 receptor, with some cross-activation of EGFR-related pathways via NF $\kappa$ B, leading to the activation of NF $\kappa$ B, which is essential in mediating cytokine expression (IL-1 $\beta$ , IL-6 and IL-8) [169]. In addition, the ability of 7KC to induce IL-1 $\beta$ -secretion suggests an activation of the inflammasome. This hypothesis is supported by several experimental studies. Thus, formation and activation of Nlrp3 inflammasomes in bone marrow derived macrophages (BMMs) has been reported in the presence of 7KC [170]. Sublethal concentrations of 7KC in retinal microglia isolated from postnatal C57BL/6J mice resulted in microglial activation and polarization to a pro-inflammatory M1 state, via NLRP3 inflammasome activation [104]. 7KC also efficiently induces inflammasome formation in fetal human RPE (fhRPE), human ARPE-19 cells, primary human brain microglia cells, and human THP-1 monocyte cells [98].

### ***2.5.2 - 7 $\beta$ -hydroxycholesterol-induced oxiaoptophagy and inflammation: associated signaling pathway***

The schematic signaling pathways presented in **Figure 3** summarize the data obtained with 7 $\beta$ -OHC on cells from different types and species: promonocytic / monocytic human U937 / THP1 cells [25, 47]; vascular cells (endothelial and smooth muscle cells) [25, 148]; wild type human mammary tumor MCF-7 cells (caspase-3 deficient), and genetically modified MCF-7 (MCF-7/c3: stably transfected with caspase-3) [138], human retinal pigment epithelial cells (ARPE-19) [100] / primary porcine retinal epithelial cells; rat C6 glioblastoma cells [171], murine oligodendrocytes 158N and murine microglial BV-2 cells [3, 114, 119, 121, 141, 142]. The different cells used permitted determination of the signaling pathways involved in 7 $\beta$ -OHC-induced cytotoxicity and to define the relationships between ROS overproduction, apoptosis, autophagy and inflammation. Distinctly from 7KC, 7 $\beta$ -OHC, while cytotoxic, does not accumulate in lipid rafts [141]. 7 $\beta$ -OHC is a strong inducer of ROS overproduction and favors the disturbance of redox homeostasis by increasing the formation of lipid peroxidation products (malondialdehyde (MDA), conjugated dienes (CDs)) and of carbonylated proteins (CPs) which can further contribute to cell death [172]. An important impact on the mitochondria was also observed whatever the cells considered. In

human monocytic THP-1, U937 cells, and MCF-7 cells, a down-regulation of Bcl-2 expression was also detected as well as an activation of the pro-apoptotic proteins (Bid, Bax), associated with a release of cytochrome c and an activation of caspase-9, caspase-8, caspase-3 and caspase-7. In U937 cells, 7 $\beta$ -OHC also induced an increase in cytosolic Ca<sup>2+</sup> concentration, associated with a decrease of Akt activation and a mitochondrial release of various proteins such as cytochrome c, apoptosis-inducing factor (AIF), and endonuclease-G (Endo-G), associated with caspase-3, -7, -8, and -9 activation, Bid cleavage and PARP degradation [25, 138]. In C6 glioblastoma cells, 7 $\beta$ -OHC induces apoptosis through decreased ERK signaling, transient PI3K / Akt activation, loss of GSK3 $\beta$  activation and activation of p38 [171, 172]. In U937 cells, as well as in human retinal pigment epithelial cells (ARPE-19), large myelin figures (evocating reticulophagy) were observed [25, 100]. In ARPE-19 cells, a link between lysosome and cell death was also established [100]. In addition to lysosomal and mitochondrial dysfunctions, in 158N and BV-2 cells, 7 $\beta$ -OHC also induces peroxisomal changes (morphological and topographical changes, reduce number of peroxisome, altered metabolism). In 158N cells, the complex mode of cell death induced by 7 $\beta$ -OHC (oxiaptophagy) is characterized by a dephosphorylation of PKB / Akt, an activation of GSK3, and by a reduced expression of Bcl-2; altogether these events contribute to mitochondrial depolarization leading to caspase-3 activation, PARP degradation and internucleosomal DNA fragmentation [141]. Moreover, 7 $\beta$ -OHC promotes the conversion of microtubule-associated protein light chain 3 (LC3-I) to LC3-II which is a hallmark criterion of autophagy [119]. The ratio [LC3-II / LC3-I] is also strongly modified by bafilomycin acting on the autophagic flux. Rapamycin, an autophagic inducer, and 3-methyladenine, an autophagic inhibitor, reduce and increase 7 $\beta$ -OHC-induced cell death, respectively, supporting that 7 $\beta$ -OHC induces survival autophagy [114]. Altogether, these data establish that 7 $\beta$ -OHC is a potent inducer of oxiaptophagy through the concomitant activation of several signaling pathways involved in oxidative stress, apoptosis and autophagy.

There is also evidence that 7 $\beta$ -OHC is a potent inducer of inflammation: it increases cytokine secretion (IL-8, IL-1 $\beta$ ) and overexpression of adhesion molecules (VCAM-1, ICAM-1, E-selectin). In U937 and THP-1 cells, the secretion of cytokines and the expression of adhesion molecules observed under treatment with 7 $\beta$ -OHC were higher than with 7KC [61, 167, 168]. Currently, no data are available on the relationship between 7 $\beta$ -OHC-induced IL-1 $\beta$  secretion and inflammasome activation. Of note, it has been described in several works that the PKC/ P38/

MEK/ ERK signaling pathway constitutes a link between  $7\beta$ -OHC-induced apoptosis and inflammation [168, 171, 172].

Comparatively to 7KC, less information are available on the  $7\beta$ -OHC. It is however well established that  $7\beta$ -OHC is a more potent inducer of apoptosis than 7KC and is also a more potent inflammatory mediator. Indeed, the percentage of apoptotic cells and the level of cytokine secretion are higher under treatment with  $7\beta$ -OHC than with 7KC. Although there are common signaling pathways for both molecules, there must also be specific pathways for each of them.

### ***2.5.3 – Contribution of $Ca^{2+}$ and $K^+$ in 7-ketocholesterol and $7\beta$ -hydroxycholesterol signaling pathways***

$Ca^{2+}$  is a universal second messenger, participating in the regulation of almost every cellular process, from fertilization to motility, gene expression and death [173]. Consequently, the ability of 7KC or  $7\beta$ -OHC to modify intracellular  $Ca^{2+}$  concentrations in diverse cell-types of the vascular wall, with a range of downstream consequences, have been studied. In HUVECs, incubation with 150  $\mu$ M 7KC for 2 h elevated resting cytosolic  $Ca^{2+}$  levels and enhanced  $Ca^{2+}$  responses to histamine [174]. In mouse aortic endothelial cells, micromolar concentrations of 7KC elicited a transient increase in cytoplasmic  $Ca^{2+}$  within seconds of addition, via a mechanism partially dependent on  $Ca^{2+}$  release from intracellular pools, since it persisted in  $Ca^{2+}$ -free extracellular medium. This transitory rise in  $Ca^{2+}$  promoted reactive oxygen species (ROS) formation, with subsequent apoptotic cell-death [175]. In contrast, low micromolar concentrations of  $7\beta$ -OHC promoted survival of HUVECs, increasing the phosphorylation of extracellular signal-regulated kinases (ERKs). These effects are probably mediated by a store-operated  $Ca^{2+}$  entry mechanism, since they are blocked by 2-aminoethyl diphenylborinate and by gadolinium ions [176]. In human aortic smooth muscle cells, within a few minutes of addition, micromolar  $7\beta$ -OHC triggered oscillations in cytoplasmic  $Ca^{2+}$  concentrations, followed by depletion of intracellular  $Ca^{2+}$  stores sensitive to thapsigargin, an inhibitor of sarco-/endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA) pumps. These changes were associated with activation of ERK1/2 within minutes and with apoptotic cell death over 72 h [177]. In the same cell type, 7KC elicited similar  $Ca^{2+}$  oscillations, enhanced activity of the NAD(P)H oxidase Nox-4, increasing ROS production and induced the ER unfolded protein response [148]. In human coronary artery smooth muscle cells, 7KC caused a rise in cytoplasmic  $Ca^{2+}$  followed by caspase-3 dependent

apoptosis. Both the ER stress response and caspase-3 activation could be partially inhibited by submicromolar concentrations of nifedipine, an antagonist of L-type voltage-gated  $\text{Ca}^{2+}$ -channels (VGCCs) [178]. In mouse coronary artery smooth muscle cells, 7KC stimulated rises in cytoplasmic  $\text{Ca}^{2+}$  that enhanced ROS formation, promoting differentiation via activation of nuclear factor erythroid 2-related factor 2 (NRF2). As revealed in smooth muscle cells from CD38<sup>-/-</sup> transgenic mice [179], these  $\text{Ca}^{2+}$  elevations were dependent on CD38, an enzyme that can generate second messengers promoting  $\text{Ca}^{2+}$  release via ER/SR ryanodine receptors (by cyclic ADP ribose), or from lysosomal  $\text{Ca}^{2+}$  stores (by nicotinic acid adenine dinucleotide phosphate) [180]. In the A7r5 rat aortic smooth muscle cell-line, incubation with 7KC or 7 $\beta$ -OHC for 24 h elevated resting cytosolic  $\text{Ca}^{2+}$  concentrations and suppressed responses to bradykinin, or arginine vasopressin, peptide hormones linked to  $\text{Ca}^{2+}$  mobilization via the phospholipase C-inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) pathway. In this system, 7 $\beta$ -OHC led to a decrease in the levels of the type 1  $\text{IP}_3$  receptor/ $\text{Ca}^{2+}$ -release channel protein, in a manner that could be abolished by co-incubation with an inhibitor of the proteasomal pathway [181]. In the THP1 monocyte/macrophage cell-line, 7KC stimulated  $\text{Ca}^{2+}$  increases that were partially inhibited by micromolar concentrations of the L-type VGCC inhibitors verapamil and nifedipine [152]. These elevations in cytoplasmic  $\text{Ca}^{2+}$  exerted diverse and opposing effects on cell-death pathways: activating calcineurin to stimulate Bcl-2-associated death promoter (BAD) [152]; causing the release of pro-apoptotic Bim-LC8 from the microtubule-associated dynein motor complex; and activating the tyrosine kinase PYK2, thereby inhibiting apoptosis via the ERK pathway [182]. In this cell-line, 7 $\beta$ -OHC also triggered  $\text{Ca}^{2+}$  influx dependent on VGCCs, promoted cell survival through the ERK pathway; and stimulated transcription and secretion of interleukin-8 [183]. In differentiated U937 cells, 7- $\beta$ OHC, but not  $\beta$ -epoxide, caused gradual increases in cytoplasmic  $\text{Ca}^{2+}$  concentration that lasted for at least 15 minutes and were inhibited by nifedipine, or by removal of extracellular  $\text{Ca}^{2+}$ . In these cells, nifedipine also reduced cell-death caused by 7 $\beta$ -OHC [184].

The molecular mechanism(s) by which 7KC or 7 $\beta$ -OHC alter cytoplasmic  $\text{Ca}^{2+}$  levels are unclear [185]. Most of these responses are too rapid to require changes in gene expression, excluding roles for liver X receptor (LXR)- or sterol regulatory element binding protein (SREBP)-dependent oxysterol sensing pathways. Cell-type and congener-specificity indicates that these effects are probably not dependent on biophysical modification of lipid membranes by oxysterols,

instead requiring specific, protein-based receptors. The intracellular oxysterol-binding protein related proteins ORP4L [186], ORP5 and ORP8 [187] modulate  $\text{Ca}^{2+}$  signaling at membrane contact sites. In radioligand binding assays using heterologous expressed proteins, 7KC displaced 25-hydroxycholesterol bound to ORP4L [188]. In hepatoma cell-lines, ORP8 is required for the cytotoxicity of various oxysterols, including that of 7KC and  $7\beta$ -OHC, demonstrating that it can act as an effector for these cholesterol oxidation products [189]. To better understand the mechanisms of intracellular  $\text{Ca}^{2+}$  modulation by 7KC and  $7\beta$ -OHC, it could be interesting to study the impact of these oxysterols on the inhibition of the catalytic subunit D8D7I of cholesterol epoxide hydrolase (ChEH) [190]. Indeed, 7KC and  $7\beta$ -OHC are potent inhibitors of ChEH [190], the subunit called  $3\beta$ -hydroxysterol- $\Delta^8$ - $\Delta^7$ -isomerase (D8D7I) has been characterized as a  $\text{Ca}^{2+}$  binding protein [191], and it is not excluded that 7KC could inhibit D8D7I activity [192]. In addition, at least two types of G-protein coupled receptor (GPR) are activated by oxysterols. The repression of the GPR Smoothened by the Sonic hedgehog peptide, thereby removing the inhibitory effect of Patched, increases cytoplasmic  $\text{Ca}^{2+}$  levels in rat gastric mucosal cells [193]. In NIH 3T3 fibroblasts, 20S-hydroxycholesterol potently activated Smoothened, but  $7\beta$ -OHC was without detectable effect [194]. A distinct GPR, Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2) or GRP183, was activated by nanomolar levels of  $7\alpha$ , 25-dihydroxycholesterol when heterologous expressed in CHO-K1 cells, causing  $\text{Ca}^{2+}$  release from intracellular stores [195]. Although  $7\beta$ -OHC is a very weak agonist of this receptor, both it and 7KC can be converted into more active congeners by the actions of 25-hydroxylase and  $11\beta$ -hydroxysteroid dehydrogenases [196]. The mechanisms by which  $7\beta$ -OHC and 7KC can influence  $\text{Ca}^{2+}$  signaling are summarized in **Figure 4**.

Of note, whereas the cells of the vascular wall display rapid  $\text{Ca}^{2+}$  responses to oxysterols, such effects were not observed on nerve cells. For example, although both 7KC and  $7\beta$ -OHC promote oxiaoptophagy in mouse 158N oligodendrocytes, they did not detectably alter cytoplasmic  $\text{Ca}^{2+}$  concentrations over a 10 minutes time period [141]. These oxysterols did not detectably alter cytoplasmic  $\text{Ca}^{2+}$  levels in the SK-N-BE neuroblastoma cell-line within 10 minutes of addition, but increased the formation of calcium deposits within 24 hours, as revealed using von Kossa staining [144].

Other mechanisms that potential link 7KC and 7 $\beta$ -OHC to Ca<sup>2+</sup> signaling have not been completely defined. These mechanisms include P2X7 purinoreceptor cation channels [97], CD38 [179], and the voltage-gated K<sup>+</sup> channel K<sub>v</sub>1.3b, which modulates membrane potential and thereby VGCC opening [144]. In human retinal cells, 7KC-induced toxicity has been reported to activate the P2X7 receptor which leads to Na<sup>+</sup> and Ca<sup>2+</sup> influx, and K<sup>+</sup> efflux [97]. Activation of P2X7 receptor triggers the formation of large nonselective membranes pores which results in inflammation through the inflammasome, oxidative stress and, ultimately, cell death especially by apoptosis [97]. In 158N cells, the voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels (Kv3.1) designed for high-frequency repetitive firing and expressed by different types of nerve cells in the CNS is affected by 7KC. Increased levels of Kv3.1b protein were shown in 158N cells under treatment with 7KC, and positive correlations between Kv3.1b levels and the intracellular K<sup>+</sup> concentration ([K<sup>+</sup>]<sub>i</sub>) were observed [144]. Under treatment with 7KC, the simultaneous increased of [K<sup>+</sup>]<sub>i</sub> and of the level of Kv3.1b along with increasing percentage cells with depolarized mitochondria, ROS overproduction and markers of death [144]. This lead to speculation that K<sup>+</sup> retention could contribute to 7KC-induced cytotoxic effects and that enhanced expression of Kv3.1b could occur as a compensatory mechanism, contributing to prevention of 7KC cytotoxicity. This hypothesis is supported by the fact that the blockage of Kv channels with 4-aminopyridine exacerbated 7KC-induced cell dysfunction on 158N murine oligodendrocytes and microglial murine BV-2 cells [143].

### **3 – Contribution of bacteria for the identification of new strategies to prevent 7-ketocholesterol-induced cytotoxicity: biodegradation of 7-ketocholesterol by bacterial cells and enzymes**

Inherent metabolic insufficiency of the human body increases with age, which can lead to the accumulation of pathogenic compounds like 7KC in senile cells, causing deleterious functions and cell death. Alternatively, utilizing exogenous microbial enzymes for targeting cytotoxic compounds enabling subsequent degradation is a promising substitute to endogenous enzymes. Microbes have long been proven to be remarkable in catabolizing toxic xenobiotic compounds like pesticides, solvents and hydrocarbons, which have found application in the remediation of polluted environments [197-199]. In general, the biodegradation process involves microbial use



of the organic compounds as a carbon/energy source, breaking down of complex organic structures, assimilation or release of the degradation products, and subsequent mineralization to carbon dioxide and water. ‘Medical bioremediation’ as a novel strategy, has been proposed to remediate cytotoxicity of 7KC which involves screening of microbes capable of catabolizing 7KC, identification of relevant enzymes, overproduction of these enzymes, and delivery into targeted organelle of diseased cells [5, 6, 200, 201]. The enzymes could also have application in food productions to remediate 7KC in its dietary sources. In fact, use of such systems against toxic molecules has been reported in amyloid- $\beta$  degradation by insulin-degrading enzyme [202] and mycoplasma cells [203]; cleavage of bisretinoid lipofuscin (A2E) by horseradish-peroxidase [204]; and hydrolysis of blood cholesterol and triglycerides by *Pseudomonas gessardii* lipase [205].

The earliest studies on 7KC degradation identified several bacterial and actinobacterial strains isolated from soil and activated sludge, namely *Nocardia nova*, *Proteobacterium* Y-134, *Sphingomonas* sp. JEM-1, *Rhodococcus jostii* RHA1 and *Pseudomonas aeruginosa* having the potential to mineralize 7KC, using it as a sole-carbon and energy source. The first four strains were also found to release CO<sub>2</sub> in minimal salt media with 0.1% 7KC [206]. Additional studies by the same group conducted transcriptomic analysis of genes expressed by *Rhodococcus jostii* RHA1 in presence of 7KC and cholesterol revealed 363 differentially expressed in presence of 7KC compared to cholesterol. In fact, 7KC was found to induce a larger number of steroid catabolism genes, mostly belonging to three or four putative clusters. The genes included those responsible for the catabolism of the steroid rings such as *kstD*, *kshA*, *hsaABCDEFGF*, or their homologs. The steroid uptake system Mce4 was also found essential for uptake of 7KC into the cell. Other genes identified include *hsaC* coding for a dioxygenase which cleaves the ring A of cholesterol and Cyp125 which initiates side-chain degradation. However, none of these clusters code for a complete 7KC degradation pathway. It seems that cholesterol and 7KC degradation follows a common pathway until the *hsaC* step and the degradation of the side chains occur simultaneously with that of the rings. Several metabolites of the pathway such as 3,4,7-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione propionic acid (3,4,7-THSAP), 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione propionic acid (3,4-DHSAP), 3,4,7-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4,7-THSA) and 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) were identified, along with the

involvement of enzymes such as dioxygenase (*hsaC*), 7-keto reductase and dehydratase [207]. Subsequent studies on *Pseudomonas aeruginosa* PseA and *Rhodococcus erythropolis* MTCC 3951 reported respectively 88% and 93% degradation, of an initial 7KC concentration of 1 g/L (1000 ppm), with a high growth of biomass. The extracellular extracts of the bacterial strains were capable of degrading 0.1 g/L (100 ppm) 7KC to a very high extent (65% in case of *P. aeruginosa* and 98% in *R. erythropolis*) within 72 hours, pointing to the involvement of enzymatic systems in the degradation process. Cholesterol oxidase was assayed as the main enzyme responsible for the first reaction pertaining to conversion of 7KC to 4-cholesten-3, 7-dione. In *P. aeruginosa*, cholesta-3, 5-dien-7-one/cholesta-4, 6-dien-3-one were found to be the next intermediate products, while in case of *R. erythropolis*, chol-5-en-3,7-dione and androsta-4-ene-3,7,17-trione were found to be formed downstream in the pathway. Here, the side-chain degradation precedes that of the ring-cleavage of 7KC. Lipase, reductase and dehydrogenase were the other identified enzymes secreted by the microbes in presence of 7KC [208, 209]. More recently, environmental samples such as soil, sea water sediment and manure piles have been explored for the presence of 7KC degrading microbes. *Alcanivorax jadensis* IP4 isolated from sea water sediment, *Streptomyces auratus* IP2 and *Serratia marcescens* IP3 isolated from soil samples and *Thermobifida fusca* IP1 isolated from manure piles proved to be potent strains capable of using 7KC (1 mg/L) as a sole carbon source and to subsequently mineralize it. Out of these, *A. jadensis* IP4, followed by *T. fusca* IP1 were most efficient in degrading 7KC, with 100% degradation of 1 mg/L concentration achieved within 12 days. Intracellular extracts of *A. jadensis* IP4 were able to degrade 65% of same concentration of 7KC within 72 hours [210, 211].

As in the case of storage diseases, bulk of the 7KC absorbed by the cell is localized to lysosomes. Thus, in spite of the reason that several enzymes such as 27-hydroxylase (CYP27A1), 11 $\beta$ -Hydroxysteroid dehydrogenase, cholesterol sulfotransferase (SULT2B1b) and Acyl-CoA cholesterol acyltransferase (ACAT) have been known to act on 7KC, their unavailability in lysosomes, prevents the attenuation of its cytotoxicity. In liver cells, 11beta-hydroxysteroid dehydrogenase type 1 converts 7KC to 7 $\beta$ -OHC, which is then transformed by the hepatic metabolism [212]. A plasmid construct of pEGFP-N3, harboring the *Chromobacterium* DS-1 cholesterol oxidase gene fused with the signal sequence and transmembrane domain of the lysosomal membrane protein LAMP1 (named as pEGFP-COXL1) was found to be localized within the lysosome. Human fibroblast cells transfected with this plasmid were able to withstand

up to 50  $\mu\text{M}$  concentration of 7KC, compared to the control, proving the cytoprotective effect of the enzyme [213]. The role of cholesterol oxidase in 7KC biotransformation was further proved in another application study, where the enzyme immobilized on magnetic Iron (II, III) oxide nanoparticles was able to convert cholesterol and 7KC to 4-cholesten-3-one and 4-cholesten-3, 7-dione respectively, which could be used as steroid precursors or anti-obesity drugs [214]. An interesting investigation reports the ability of growing and resting cells of *Lactobacillus casei* ATCC334 in removing oxysterols from solution, probably through binding to the cell surface or by membrane incorporation. The resting cells were most efficient in removal (37 to 61%) of oxysterols such as 7KC followed by  $7\alpha/7\beta$ -OHC, cholestanetriol, 5,6 $\beta$ /5,6 $\alpha$ -epoxycholesterol and 25-hydroxycholesterol respectively. Being probiotics, *L. casei* may find application in inhibiting the intestinal absorption of harmful oxysterols [215].

Thus, microbial biodegradation and consequent mining of therapeutic enzymes may provide a promising route to remediate oxysterol-mediated cytotoxicity, especially that of 7KC and  $7\beta$ -OHC.

#### **4 – Animal models and analysis of the biological activities of 7-ketocholesterol and $7\beta$ -hydroxycholesterol**

Currently, the biological activities of 7KC and  $7\beta$ -OHC have mainly been evaluated in animal models developed to study atherosclerosis. In this context, numerous animal models are available. At the opposite extreme, only a few animal models are described to determine the incidence of these oxysterols in eye, neurodegenerative and bowel diseases.

##### **4.1 – Mouse transgenic models**

To address the incidence of oxysterols in different pathologies, murine transgenic animal models have been developed when the relevant oxysterols considered are enzymatically produced. This is mainly the case for oxysterols oxidized on the lateral chain, which do not include 7KC and  $7\beta$ -OHC. Indeed, the oxysterols oxidized on the lateral chain interact with the Liver X Receptors (LXR $\alpha/\beta$ ) [216]. LXR deficient transgenic mice have contributed to demonstrate the importance of LXR in the fertility and development of prostate cancer) [217-219]. LXR $\beta$  is also involved in

amyloidogenesis associated with neurodegeneration in Alzheimer's disease [220]. On the other hand, CYP46A1 is mainly expressed in the central nervous system neurons and allows the production of 24S-hydroxycholesterol (24S-OHC) [221]. The use of CYP46A1-deficient mice permitted to demonstrate the importance of this enzyme and 24S-OHC in major neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease [222-225]. As for the enzyme CYP27A1 which makes it possible to produce 27-hydroxycholesterol (27-OHC) from cholesterol essentially at the peripheral level, the use of CYP47A1 deficient mice has demonstrated the importance of this enzyme and of 27-OHC in cancer development (breast cancer, melanoma) [226, 227].

Unlike oxysterols oxidized on the lateral chain such as 24S-OHC and 27-OHC, 7KC and 7 $\beta$ -OHC are essentially formed by auto-oxidation of cholesterol in different tissues (vascular wall, retina, brain) under the influence of an environmental oxidative stress [25]. They also accumulate in the vascular wall, and this process is favored by a diet rich in oxysterols even if these latter are metabolized by the liver [25].

ApoE deficient mice were used to determine the vascular effects of 7 $\beta$ -OHC [228]. CX3C chemokine receptor 1 (CX3CR1<sup>GFP/+</sup>) mice were also successfully used to demonstrate that retinal microglia have a prominent chemotropism to 7KC and internalize 7KC. Sub-lethal concentrations of 7KC resulted in microglial activation and polarization to a pro-inflammatory M1 state via NLRP3 inflammasome activation. In addition, microglia exposed to 7KC reduced expression of neurotrophic growth factors but increased expression of angiogenic factors, transitioning to a neurotoxic and pro-angiogenic phenotype [104]. Since the enzyme 11 $\beta$ -HSD11B1 has been shown to convert 7KC to 7 $\beta$ -OHC, mouse models deficient for this enzyme, that also converts cortisone to cortisol, have been developed to study atherosclerosis and metabolic syndrome [36, 37, 229-231]. In the context of obesity (with or without metabolic syndrome, and type 2 diabetes), the role of 11 $\beta$ -HSD11B1 and 11 $\beta$ -HSD11B2 in ob/ob mice is a subject of new research [232, 233]. Mice deficient in the enzyme 7-dehydrocholesterol reductase used, as a model of Smith-Lemli-Opitz syndrome, are also available to address the conversion of 7-dehydrocholesterol to 7KC in this disease [234]. In addition, ApoE-deficient mice are of interest in clarifying the role of 7KC and 7 $\beta$ -OHC in atherogenesis [84].

#### **4.2 – Non transgenic invertebrate and vertebrate animal models**

Due to the identification of 7KC and 7 $\beta$ -OHC in large quantities in different types of foods, the *in vivo* effects of these molecules have been studied in animal models, in order to determine their effects on diseases associated with poor eating habits, such as cardiovascular diseases. To this end, several models (pigeon, mouse, rat, hamster, rabbit and pig) were used (**Figure 5**). Among these models, some of them were also employed to study the impact of 7KC in aged related diseases, and in this context the *Caenorhabditis elegans* model was recently developed.

#### 4.2.1. *Caenorhabditis elegans*

With the use of the nematode *Caenorhabditis elegans* as model organism, the toxicity of 7KC was investigated [235]. The effects of 7KC on life span, on reproduction, thermotolerance, germline apoptosis, and ROS generation resulting from *C. elegans* exposure to 7KC were investigated at concentrations ranging from 0 to 200  $\mu\text{g/mL}$ . In these conditions, 7KC reduced reproductive capacity, shortened the life span in a concentration-dependent manner, and impaired thermotolerance of the adult nematode. 7KC also induced germline apoptotic cell death and increased ROS generation. It is suggested that the model *C. elegans* could be suitable for assessment of the bioactivity of 7KC in aging.

#### 4.2.2. Pigeon

The pigeon model has been one of the first to examine the effects of tobacco smoking and of a diet enriched in cholesterol on the development of atherosclerosis [236, 237]. The toxicity of oxysterols was also studied in experiments performed on White Carneau pigeons. When the pigeons were fed with a diet supplemented with 0.05% pure cholesterol (control group); 0.05% pure cholesterol plus cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ - triol (which can be present in powdered milk in quantity as high as 7KC [238], total aortic cholesterol, aortic cholesterol ester, and the ratio (aortic cholesterol ester / aortic cholesterol) were similar among pigeons from both groups, whereas an accumulation of calcium in the aortas of pigeons fed with cholesterol plus cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ - triol was observed [239].

#### 4.2.3. Golden Syrian hamster

Syrian hamster is a widely used experimental pharmacological model to identify natural and synthetic anti-atherosclerotic drugs [199, 240-244]. In addition, the effect of dietary oxysterols on

coronary atherosclerosis was also studied in golden Syrian hamsters fed for 3 months with three different diets: a normolipidaemic diet containing corn oil plus fish oil (group low L); a hyperlipidaemic diet composed of the normolipidaemic diet supplemented with cholesterol (group High L); a third diet, similar to the hyperlipidaemic diet, in which cholesterol was replaced by a mixture of oxysterols: 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol, 5 $\beta$ ,6 $\beta$ -epoxycholesterol, 7 $\alpha$ -hydroxycholesterol, 7 $\beta$ -OHC, 7KC and trace amounts of 7-hydroperoxycholesterols (group High L + OS) [245]. Feeding the high-lipid diet (group High L) increased the plasma level of 7 $\beta$ -OHC, 7KC and cholestanetriol. The presence of oxysterols in the diet (group High L+OS) further increased the concentrations of 7 $\beta$ -OHC and 7KC in the plasma. 7KC was increased in myocardial lipids of groups (High L) and (High L+OS). However, as evidenced by myocardial Ca<sup>2+</sup>, acyl-CoA cholesterol acyl transferase (ACAT) activity and coronary reactivity to sodium nitroprusside, severe atherosclerosis did not develop during the 3-month enriched lipid diet.

#### 4.2.4. Mouse

Wild type mice treated with oxysterols, which are oxidized on the side chain or steroid nucleus, are used to mimic inflammatory bowel diseases [2]. In mice, 7KC and 7 $\beta$ -OHC are often incorporated into a mixture of oxysterols (7KC, 5 $\alpha$ ,6 $\alpha$ -epoxicholesterol, 5 $\beta$ ,6 $\beta$ -epoxicholesterol, 7 $\alpha$ -hydroxycholesterol and 7 $\beta$ -OHC) to mimic food leading to inflammation of the intestinal wall to identify cytoprotective molecules; in the mouse, this oxysterol mixture induces TLR-2 and TLR4 over-expression and activation together with cytokine induction [128].

Anti-tumor properties of 7KC have also been described in mice [246] as well as anti-tumor activity of 7 $\beta$ -OHC against Krebs II ascitic carcinoma transplanted on mice [247]. Anti-tumor activity of the water-soluble monophosphoric acid diesters of 7 $\beta$ -OHC on mastocytoma P815 in the mouse model has also been reported [248]. The pioneering work realized by Bischoff *et al.* concerning the cytotoxic and anti-tumor activity of some oxysterols, including 7 $\beta$ -OHC, lead to the suggestion that some oxysterols could constitute potent anti-tumoral molecules [249]. There are now several lines of evidence that 7KC and 7 $\beta$ -OHC have major impacts on the metabolism of cancer cells [250], supporting the concept that chemotherapies targeting metabolism offer promising perspectives for cancer treatment.

#### 4.2.5. Rat

The rat model has been used to study the metabolism of 7KC [26, 50, 251], to identify molecules capable of preventing cardiovascular injuries [252] and to determine the impact of some nutrients and aliments on the biogenesis of oxysterols formed by auto-oxidation including 7KC and 7 $\beta$ -OHC [253-255]. In addition, using 7KC-containing implants inserted into the anterior chamber of the rat eye, it has been demonstrated that the cytokinic inflammation induced by 7KC mostly occurs through the TLR4 receptor [169]. A significant increase in vascular endothelial growth factor (VEGF) was also observed in the rat eye with 7KC-containing implants by fluorescent immunolabeling and by immunoblot of the aqueous humor [256]. To define the role of 7KC in Smith-Lemli-Opitz syndrome, intra-vitreous injection of 7KC into a normal rat eye rapidly induced panretinal degeneration [257]. Strong impacts of 7KC and 7 $\beta$ -OHC were also observed when these compounds were injected into the rat prefrontal cortex, based on the analysis of RNA extracted from this brain region at 24 hours post-injection [258]. Microarray analyses identified 1365 genes, whose expression were affected by these two oxysterols: down-regulated genes outnumbered up-regulated genes. Pathway analysis showed that down-regulated genes had roles in carbohydrate metabolism, cell signaling and nucleic acid metabolism; and that the majority of these encode G-protein coupled receptors (GPRs) involved in the synaptic function [258]. On tumors induced by C6 cells in the rat brain cortex, it has also been reported that the intra-tumoral injection of liposomes containing 7 $\beta$ -OHC ether or ester inhibited tumor growth [259].

#### 4.2.6. Rabbit

The rabbit is an animal model used for several years for the study of atherosclerosis [260, 261]. One of the oldest studies to address the *in vivo* toxicity of 7KC was conducted in the rabbit in 1949 by Cox and Spencer [262]. It was also established in the rabbit that 7KC inhibits cholesterol uptake by the arterial wall, and that this oxysterol can bind to the lipoproteins [263, 264]. In 1980, experimental evidence of the toxicity of 7KC were described in the rabbit: it was shown that the rate constant determining tissue uptake of 7KC was higher than tissue efflux, and suggested that the red cells and peripheral tissues act as a reservoir for the oxygenated sterol [265]. The rabbit is also a source of vascular cells and pioneering works supporting cytotoxic effects of 7KC and 7 $\beta$ -OHC were performed on cultured rabbit aortic smooth muscle cells [266]. In addition, *ex-vivo* experiments on rabbit aortic segments demonstrated that 7KC and 7 $\beta$ -OHC were able to prevent arterial relaxation [267].

#### 4.2.7. Pig

The domestic pig and mini-pig are conventional models of study in pharmacology, imaging and cardiovascular surgery [74, 268]. The pig is often a source of vascular wall and ocular cells that have made it possible to characterize the effects of different oxysterols, such as 7KC and 7 $\beta$ -OHC. In smooth muscle cells of porcine aorta, it has been shown that the cytotoxicity of oxidized Low Density Lipoprotein (LDL) was associated with 7KC and 7 $\beta$ -OHC [102, 269]. The use of pig in the mechanical ventilation of the lungs made it also possible to establish that 7KC was probably an important player in pulmonary inflammation associated with respiratory support [270].

#### 4.2.8 Monkey

11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) catalyzes the conversion of cortisone to cortisol and controls a key pathway in the regulation of stress [271]. 11 $\beta$ -HSD1 also converts 7KC to 7 $\beta$ -OHC; these two oxysterols are at increased levels in the brain of Alzheimer's patients [110, 272]. F18-radiolabeled ligands have been developed to determine the topographical expression of 11 $\beta$ -HSD1 in the brain of rhesus monkey (*Macaca mulatta*) [273]. The authors had previously developed [<sup>11</sup>C] AS2471907 PET radiotracer for imaging 11 $\beta$ -HSD1 in the brain, but their syntheses were not reliable, so they developed new probes and optimized the synthesis. Pharmacokinetic analyses and verification of the binding specificity of the new probes were performed in monkeys. The developed probes have a heterogeneous distribution, binding specificity and longer half-life than the previous developed probes as well as easier production, making them efficient and suitable PET radiotracer for 11 $\beta$ -HSD1 brain imaging.

### 5 - Alternatives to animal models for the study of 7-ketocholesterol- and 7 $\beta$ -hydroxycholesterol: organoids and microfluidic associated technologies

There are now evidence that whatever the relevance of models used to evaluate the biological activities of natural or synthetic compounds, it is difficult to anticipate their biological activities in humans. So, there is a need to develop new models including several parameters and mimicking the complexity of an organ or of the whole organism. These approaches constitute an important biotechnological challenge to prevent and cure major diseases (such as cardiovascular



diseases and neurodegeneration), where no efficient treatments are available. These new cell culture technologies include organoids, as well as organ-on-a-chip and multi-organ microphysiological system models, also named body on-a-chip, based on a microfluidic technology [274].

- ✓ Organoids mimicking the respiratory system, bowel or brain have already been made and can be produced from various sources such as primary cells [275], pluripotent stem cells [276, 277], embryonic [278] or adult stem cells [279], and patient-derived induced pluripotent stem cells [276, 280]. Although several studies have been carried out using established cell lines or primary cells, the use of stem cells is increasing because of the tremendous potential to model various disease models or biological systems.
- ✓ Organ-on-a-chip strategies are based on microfluidic cell systems to model physiological functions of tissues, or organs. To this end different types of stem cells can be used. Currently, the focus is not to rebuild a whole living organ, but to mimic minimal functional units that recapitulate tissue and organ level functions. Precise control of stem cell differentiation in the microfluidic microenvironment makes tissue engineering and organ-on-a-chip developments highly promising [281]. To date, a number of proof-of-concept, organ-on-a-chip systems using cells differentiated from stem cells have been described [282, 283]. Patient-derived and genetically engineered iPSCs with tissue engineering to elucidate the pathophysiology underlying the cardiovascular diseases have been combined through 'heart-on chip' for modeling the mitochondrial cardiopathy of Barth syndrome [284]. These microfluidic organ-on-a-chip models can recapitulate important organ-level functions, multicellular microarchitecture, and environment dynamics. Therefore, the engineered novel heart- and vasculature-on-a-chip systems could contribute to the development of suitable high-throughput platforms for drug development and disease modeling of major cardiovascular diseases [285]. Furthermore, organ-on-a-chip platforms have also the potential to strongly impact and improve the drug screening process for neurodegenerative diseases. Patient-derived neurons from different regions of the brain can be directly grown and differentiated on a brain-on-a-chip device in where the disease development, progression and pharmacological treatments can be studied and monitored in real time for Alzheimer's and Parkinson's diseases [286-288].
- ✓ Personalized multi-organ microphysiological system models, body on-a-chip, based on

patient-derived iPSCs represent also a promising approach to elucidate physiopathology and therapies [282, 289]. The technology of multi-organs can mimic complex biological processes involving organ-organ interaction, system homeostasis and pharmacokinetics [290, 291].

These organoids and on chip models based on microfluidic technologies could be very useful to study 7KC- and 7 $\beta$ -OHC-induced biological activities on different organs and to identify new molecules and new strategies capable to prevent their side effects.

## 6 – Conclusion

The potential implications of 7KC and 7 $\beta$ -OHC in many common and disabling diseases (cardiovascular diseases, cataract, ARMD, neurodegenerative diseases, inflammatory bowel diseases) are well documented. However, the demonstration of direct or indirect involvement of these molecules in these diseases still requires significant work on cells, animal models and / or alternatives to animal models. These different approaches will make it possible to better understand the biological activities of 7KC and 7 $\beta$ -OHC, and to identify natural or synthetic molecules, or mixtures of molecules, capable of preventing their deleterious effects in fatal and/or strongly debilitating diseases, with important societal impacts.

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## Conflict of interest

The authors have no conflict of interest to declare.

## Figure Legends

**Figure 1: Oxysterols oxidized at C7.** Oxysterols oxidized at C7 ( $7\alpha$ -hydroxycholesterol,  $7\beta$ -hydroxycholesterol, and 7-ketocholesterol (also named 7-oxocholesterol)) are formed either by auto-oxidation of cholesterol or enzymatically:  $7\alpha$ -hydroxycholesterol is formed via the enzyme CYP7A1;  $7\beta$ -hydroxycholesterol and 7-ketocholesterol are formed by auto-oxidation. 7-ketocholesterol can be converted in  $7\beta$ -hydroxycholesterol via the enzyme 11 $\beta$ -HSD1 (the enzyme works less efficiently to convert  $7\beta$ -hydroxycholesterol in 7-ketocholesterol) and  $7\beta$ -hydroxycholesterol can be converted in 7-ketocholesterol via the enzyme 11 $\beta$ -HSD2 [41, 292].

**Figure 2: Signaling pathways associated with 7-ketocholesterol-induced oxiaoptophagy and inflammation.** The schematic signaling pathways associated with 7KC-induced oxiaoptophagy and inflammation are obtained from cells of different types and of different species. 7KC, which accumulates in lipid rafts and triggers phosphatidylserine externalization, modulates the activity of  $Ca^{2+}$ ,  $K^+$  and  $Na^+/K^+$  channels such as P2X7 and Kv3.1 receptors. This favours an intracellular accumulation of  $Ca^{2+}$  and  $K^+$ . 7KC also activates endoplasmic reticulum stress and oxidative stress (ROS overproduction) which contributes to organelle dysfunctions (mitochondria, peroxisome, lysosome). In addition, the ability of 7KC to inhibit the PI3-K/PKB/Akt signaling pathway participates to the loss of transmembrane mitochondrial potential ( $\Delta\Psi_m$ ). Altogether, these different effects induce autophagy and apoptosis. The simultaneous induction of oxidative stress, apoptosis and autophagy is defined as oxiaoptophagy. 7KC is also a pro-inflammatory molecule which triggers inflammation via the activation of TLR4 receptor.

**Figure 3: Signaling pathways associated with  $7\beta$ -hydroxycholesterol-induced oxiaoptophagy and inflammation.** The schematic signaling pathways associated with  $7\beta$ -OHC-induced oxiaoptophagy and inflammation are obtained from cells of different types and of different species.  $7\beta$ -OHC, which does not accumulates in lipid rafts, favors phosphatidylserine externalization. It also favors an intracellular accumulation of  $Ca^{2+}$ , and inhibits the PI3-

K/PKB/Akt signalling pathway; this later participates to the loss of transmembrane mitochondrial potential ( $\Delta\Psi_m$ ). In addition,  $7\beta$ -OHC is also a potent inducer of oxidative stress (ROS oversproduction; enhanced levels of conjugated dienes (CDs), malondialdehyde (MDA) and carbonylated proteins (CPs)) which contributes to organelle dysfunctions (mitochondria, peroxisome, lysosome) and to the induction of cell death defined as oxiaoptophagy. As  $7\beta$ -OHC and 7KC are two potent inducers of oxiaoptophagy some signaling pathways are similar. However, as  $7\beta$ -OHC is a stronger inducer of apoptosis and inflammation than 7KC, this suggests some differences between these two oxysterols. In addition, the activation of the PKC/ P38/ MEK/ ERK signaling pathway constitutes a link between  $7\beta$ -OHC-induced apoptosis and  $7\beta$ -OHC-induced inflammation allowing thus to rely oxiaoptophagy and inflammation.

**Figure 4 Mechanisms by which 7-ketocholesterol and  $7\beta$ -hydroxycholesterol can modulate  $Ca^{2+}$  signal transduction in mammalian cells.** Both oxysterols (7KC,  $7\beta$ -OHC) can be metabolized to  $7\alpha$ , 25-hydroxycholesterol, a potent agonist of the G-protein coupled receptor EBI-2/GPR183 that mobilizes  $Ca^{2+}$  from the endoplasmic reticulum (ER) by gating inositol 1,4,5-trisphosphate receptors ( $IP_3R$ ). The enzyme CD38 is activated by 7KC or  $7\beta$ -OHC via an undetermined mechanism, generating the second messengers cyclic ADP ribose (cADPr), stimulating  $Ca^{2+}$  release from the ER or smooth reticulum via ryanodine receptor (RyR) channels, and nicotinic acid adenine dinucleotide phosphate (NAADP), that mobilizes  $Ca^{2+}$  from the lysosome via transient receptor potential mucolipin (TRPML) and two-pore (TPC) channels. Both 7KC and  $7\beta$ -OHC also promote  $Ca^{2+}$  influx by activating multiple families of plasmalemmal cation channels: voltage-gated calcium channels (VGCC); store-operated calcium channels (SOCE); transient receptor potential (TRP) channels; and the purinoceptor 2X7 (P2X7). By increasing the levels and function of potassium channels such as  $K_{v1.3b}$ , oxysterols alter membrane potential, thereby modifying the gating of VGCCs.

**Figure 5: Animal models used to evaluate the biological activities of 7-ketocholesterol and  $7\beta$ -hydroxycholesterol.** Among these models transgenic and wild type mice have been widely used. The other animal models mainly concern wild type animals.

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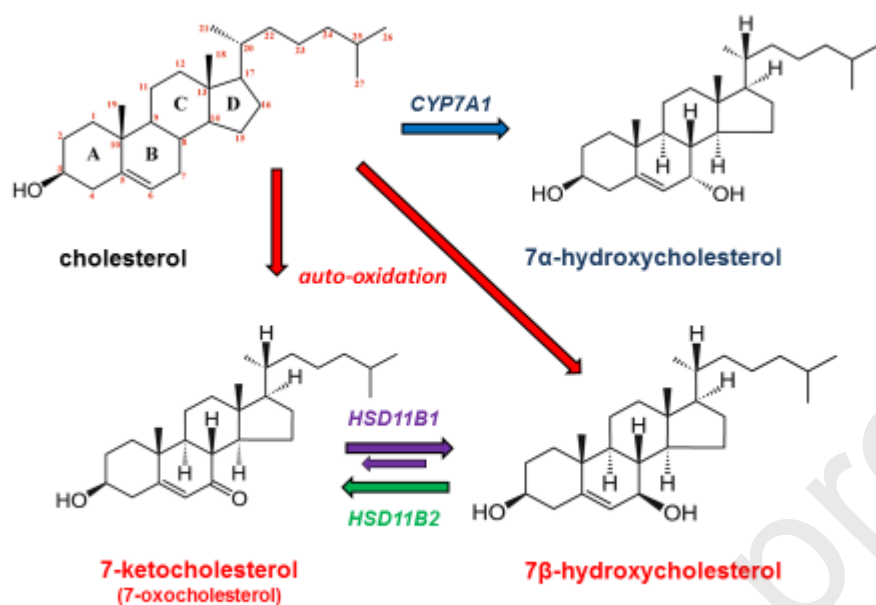


Figure 1 – Vejux et al.

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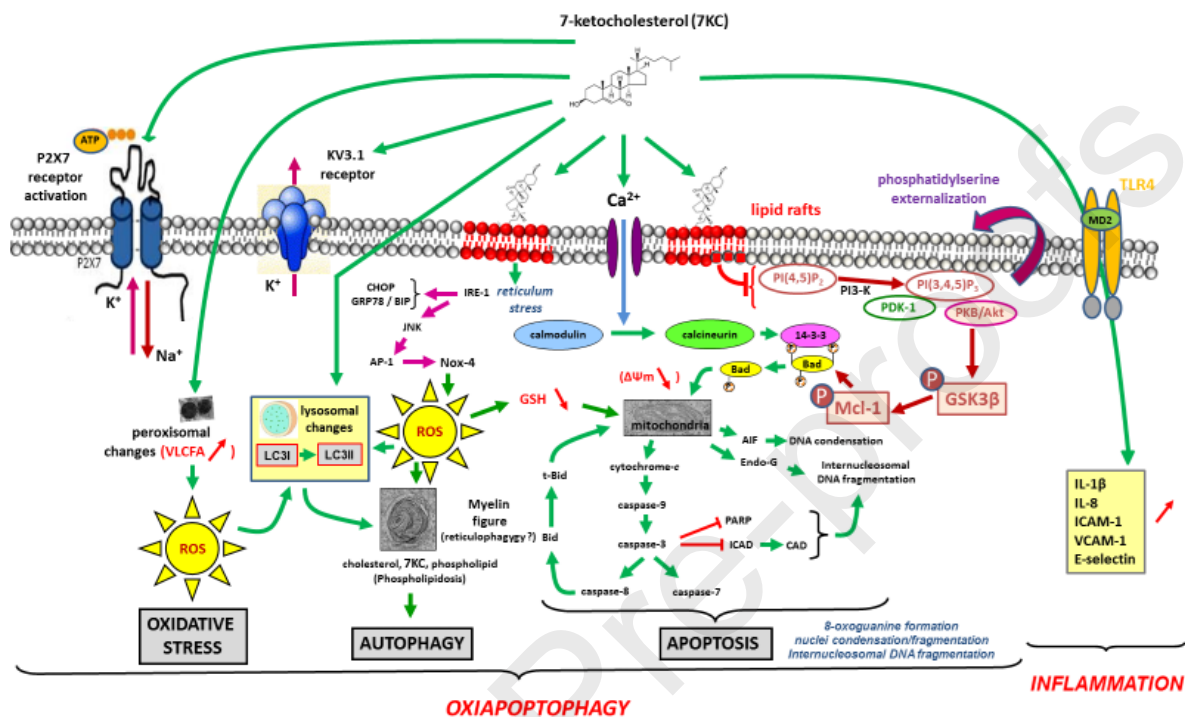


Figure 2 – Vejux et al.

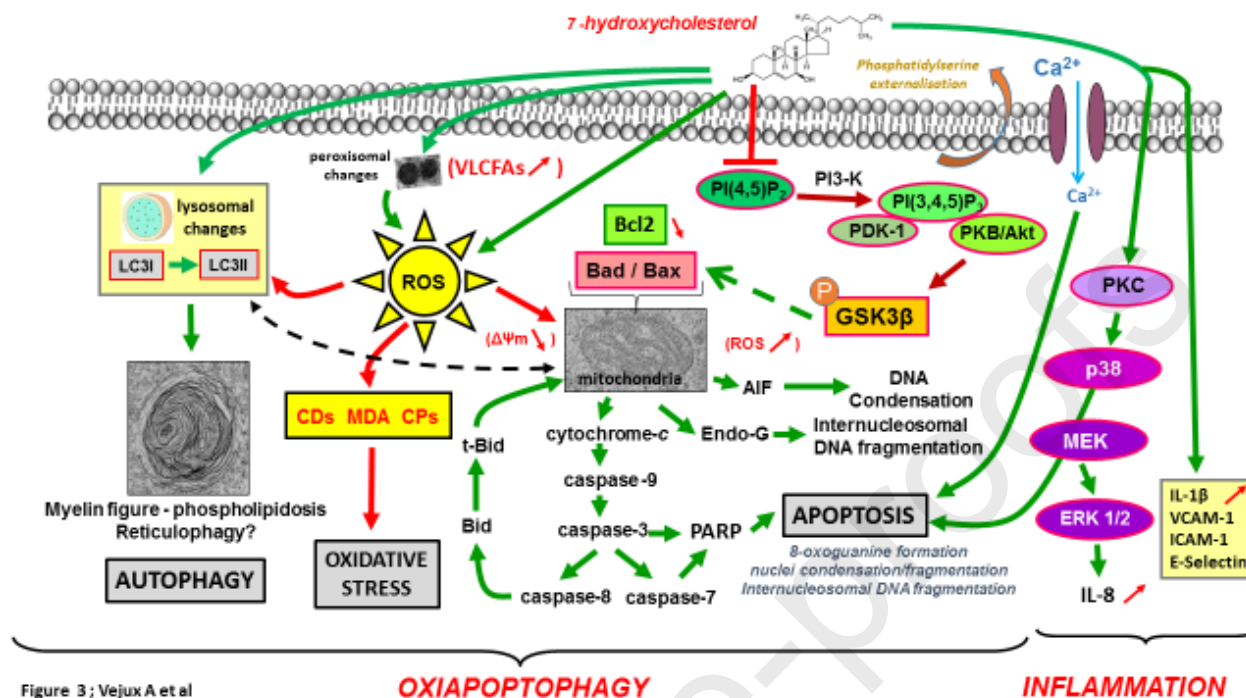
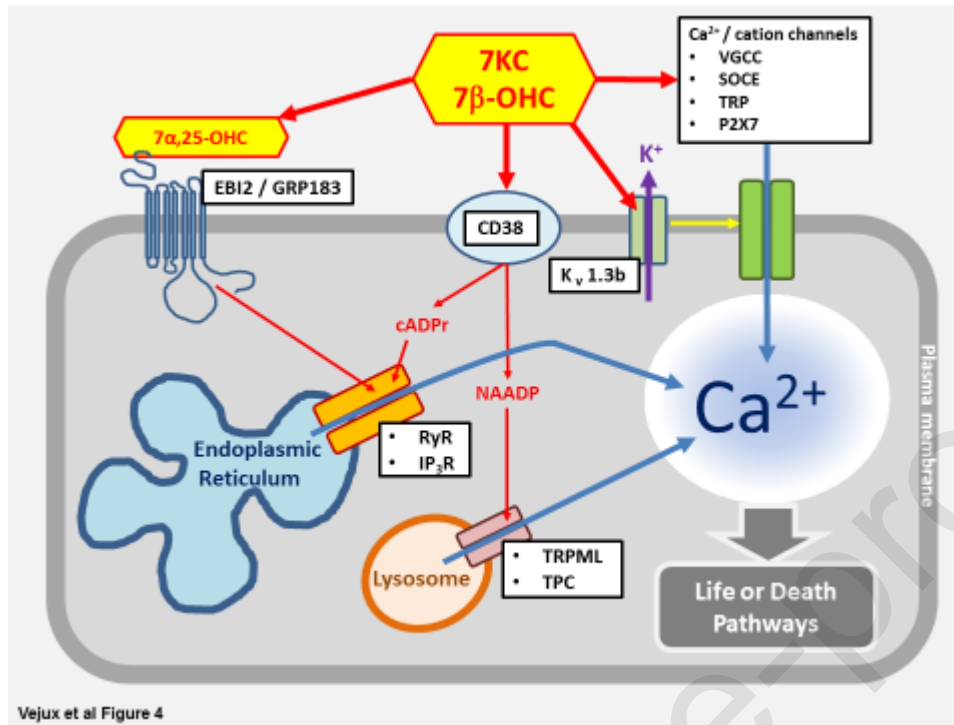


Figure 3 ; Vejux A et al

**OXIAPOPTOPHAGY**

**INFLAMMATION**

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VEJUX et al Figure 4

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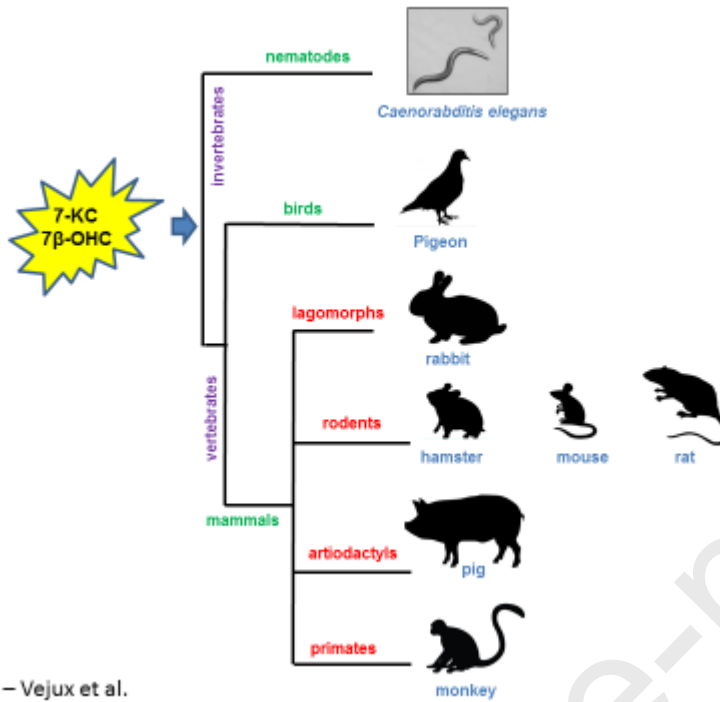


Figure 5 – Vejux et al.

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